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## Introduction

The eukaryotic cell cycle is regulated primarily at two points, in G1 prior to entry into S-phase and in G2 prior to entry into mitosis. The commitment to a round of cell division is made at a point in G1, referred to as the restriction point in mammalian cells<sup>(1-4)</sup> or START in yeast<sup>(5)</sup>. Passage through the restriction point depends critically on mitogen signals, but once this point is passed, cells are committed to S-phase and the remainder of the cycle in a mitogen independent manner<sup>(4)</sup>. Passage through the restriction point is thought to be the primary event controlling cell proliferation. Therefore, elucidating how positively and negatively acting genes function to regulate the G1/S transition and how mutations in these genes disrupt normal cell cycle control has been a primary focus of cancer research. Central to this focus has been the investigation of the role of cyclin-dependent kinases (Cdk) in the control of cell proliferation.

**Cyclins, Cdks, and positive growth control.** Cdks are protein kinases that require association with cyclins and phosphorylation for activity<sup>(5-8)</sup>. Cyclins promote cell cycle transitions via their ability to associate with and activate their cognate Cdks<sup>(5-12)</sup>. Cyclins D and E function in G1<sup>(6, 10, 13-16)</sup>, and overexpression of cyclin D1 or cyclin E shortens G1 and accelerates entry into S-phase<sup>(1, 3, 17, 18)</sup>. Amplification of cyclins, D1, D2 and E have been identified in several tumors<sup>(19-23)</sup>. Cyclin D1 was identified as the PRAD1 oncogene<sup>(24)</sup>. Cyclin A was identified as the site of integration of HBV in a hepatocellular carcinoma<sup>(25)</sup>. Taken together, these observations suggest that inappropriate activation of Cdks is a mechanism that cells frequently use to reach the oncogenic state.

Cyclins D1, D2, and D3 bind Cdk4 and Cdk6 kinases and can phosphorylate and inactivate Rb<sup>(6, 26-29)</sup>. Because D-type cyclins are required for proliferation only if cells have an intact Rb gene, it is thought that Rb inactivation is their primary role. Cyclin E binds to and activates Cdk2 and considerable evidence has accumulated indicating that cyclin E/Cdk2 is the primary kinase involved in the G1/S transition<sup>(14, 15, 30-33)</sup>. In addition, a close homolog of Cdk2 - Cdk3 - is also thought to play a unique role in the G1/S transition<sup>(31)</sup>. Cyclin A binds Cdk2 and Cdc2 and is required for both S-phase and the G2/M transition<sup>(34-36)</sup>, while cyclin B/Cdc2 complexes appear to be specific for control of mitotic entry.

Although Cdks are thought to be the critical regulators of cell proliferation, little is known about how cyclin/Cdk complexes regulate cell proliferation during development. In this regard, we have performed an analysis of the expression of the major cyclins during mouse embryonic development and in adult tissues. We have discovered that general cyclins E, A, B, and F are expressed in all proliferating tissues while the D cyclins are distributed in a pattern distinct for each cyclin but which is a subset of the general cyclins (Parker, Harper and Elledge, unpublished results). This is consistent with the notion that D cyclins are the primary initiators of cell cycle entry and orchestrate development. We have recently observed that cyclin D1 is the only D-type cyclin induced when breast cells proliferate during pregnancy. We and our collaborators in the Weinberg laboratory discovered that development of the breast during pregnancy is dependent upon cyclin D1<sup>(37)</sup>. As mentioned above, amplification of D-type cyclins is frequently observed in breast cancer. This provides a link between development and cancer and indicates that the developmental history of the breast is relevant to its susceptibility to tumorigenesis.

Since the controls utilized during development to regulate cell proliferation are similar to those utilized in maintenance of the non-proliferative state in differentiated tissues, it is likely that these controls are reactivated or overcome in cancer. Another example of this comes from our observation that cyclin D1 is expressed at extremely high levels in the retina and is required for its development<sup>(37)</sup>. Presumably the inability to properly develop the retina in cyclin D1 mutants reflects an inability to overcome Rb. In what is clearly more than a coincidence, the retina is the same tissue in which high frequency tumors arise in Rb mutant humans. It is therefore likely that the Rb protein is important in both development of that tissue and its maintenance in the non-proliferative state. Our understanding of the links between development and cancer is in its infancy and is an area in which there is a great need to increase our knowledge base.

**Tumor suppressor proteins and negative growth control.** Rb and p53<sup>(29, 38)</sup> are the most well understood tumor suppressors. Mutations in these are found frequently in many human cancers<sup>(39,40)</sup>, and reintroduction of wild-type genes into p53<sup>-</sup> or Rb<sup>-</sup> tumor cells can suppress the neoplastic phenotype suggesting that loss of function of these genes contributes to tumorigenesis<sup>(28, 41, 42)</sup>.

Mutations in p53 are the most common lesions observed in human malignancies, occurring in greater than 50% of all tumors<sup>(39)</sup> including those of the breast. The percentage is much higher if loss of p53 function via association with viral oncoproteins (E1B of adenovirus and E6 of papilloma virus) or amplification of the p53 binding protein MDM2 are included<sup>(43)</sup>. p53 deficient mice are prone to the spontaneous development of a variety of tumor types<sup>(44)</sup>. Cellular responses to DNA damage such as apoptosis and the G1 checkpoint are dependent on p53<sup>(45-53)</sup>. p53 also controls a spindle checkpoint and prevents genetic alterations such as gene amplification<sup>(54, 55)</sup>. p53 regulates the expression of p21<sup>CIP1</sup>, an inhibitor of G1-cyclin/Cdks, in response to DNA damage<sup>(56-60)</sup>. Using a p21 knockout mouse, we have determined that p21 is required for full function of the G1 checkpoint in response to  $\gamma$ -irradiation, although there is residual checkpoint function<sup>(56)</sup>. Furthermore, these mice do not show the high rate of spontaneous tumor formation seen in p53-deficient mice. It is not clear whether p53's role in oncogenesis is through its checkpoint or apoptotic deficiencies, or a combination of these.

The current view of the role of Rb in the cell cycle is that hypo-phosphorylated Rb functions during G1 in part to block the activity of E2F and related transcription factors that are required for the expression of genes involved in S-phase<sup>(29)</sup>. Hyper-phosphorylation of Rb or association with DNA tumor virus oncoproteins such as E1A results in release of E2F and is correlated with passage into S-phase.

The above observations are consistent with a model in which increased cyclin/Cdk activity in tumors, whether by increased cyclin expression or decreased negative regulation, can overcome the cell cycle repression function of Rb via direct phosphorylation and inactivation of its growth inhibitory function. Rb therefore acts as a potential energy barrier in the pathway that cyclin/Cdks must overcome to activate cell cycle entry. Removal of the barrier (Rb) may reduce the levels of kinase activity required, but some Cdk kinase activity is still required for the process of DNA replication and can therefore act as a target of further negative regulation. In this model, p53 acts to reduce the frequency of mutations that lead to altered growth control and to kill cells that have undergone extensive damage or are inappropriately growing. To fully understand this aspect of cancer, cell cycle dysfunction, it is imperative that we have a complete understanding of the regulation of cyclin dependent kinases and their regulators in the tissues of interest.

**The cell cycle and development: potential roles for Cdk inhibitors.** Once proliferation and morphogenesis have constructed a particular structure, it is of paramount importance that the proliferative state cease and be replaced with a homeostatic state. While much attention has been focused on how cells enter the cell cycle, little is known concerning the strategies organisms employ to exit the cycle and maintain the non-proliferative state. This state is of great importance to an organism because the vast majority of its cells exist in a non-proliferative state throughout adult life. The inability to appropriately halt growth can lead to malformation during development, and to cancer. Thus, equally important in the execution of developmental programs is the arrest of growth once the program is complete. While the control of terminal differentiation promises to be complex, cell cycle arrest via inactivation of Cdks is likely to be a central feature. Recently a new class of Cdk regulatory molecules have emerged that are potential mediators of cell cycle exit and maintenance of the non-proliferative state. These are the inhibitors of cyclin-dependent kinases, CKIs. Currently two structurally defined classes of CKIs exist in mammals that are exemplified by p21<sup>CIP1</sup> (57-60) and p16<sup>INK4/MTS1</sup> (61-65).



### **Cyclin-dependent kinase inhibitors: mediators of negative cell cycle control.**

Cdk inhibitory proteins are a group of proteins that associate with and inhibit Cdks. These versatile molecules have potential roles in cell cycle arrest, checkpoint function and development and are likely to cooperate with Rb, p53, and other negative regulators in maintaining the non-proliferative state throughout adult life. At the time of submission of this grant in December 1993, the first mammalian Cdk inhibitors p21<sup>CIP1</sup>/WAF1 (57-60) and p16<sup>INK4a</sup> (61) had only recently been identified. Subsequently, we and others identified additional inhibitors including p27, p57, p15, p18, and p19 (refs 61-69). We identified p21<sup>CIP1</sup> in a two-hybrid screen designed to identify proteins that associate with Cdk2 (57). Importantly, this protein was simultaneously cloned by several other laboratories. p21 was cloned as a p53 activated gene by the Vogelstein laboratory (59), as a Cdk associated protein by the Beach laboratory (58), and as an S-phase inhibitory cDNA in senescent cells (60). Since then we and others have identified two other members of the p21 family, p27 and p57. p57, also known as KIP2 has been the focus of this study. It is expressed in the breast and is localized to 11p15.5, a locus involved in breast cancer (see below).

**Involvement of 11p15.5, the location of KIP2, in human cancers including cancer of the breast.** Several chromosomal regions show frequent loss of heterozygosity (LOH) in breast tumors including but not exclusively 3p, 7q31, 11p15, 11q13 and 17p (reviewed in 70). The chromosomal location of KIP2, 11p15.5, marks it as a candidate tumor suppressor gene of the breast. The involvement of 11p15 in the breast is well documented (70). 35% of breast tumors show LOH at 11p15.5 (119) and this LOH is associated with poor prognosis. Furthermore, 11p15 LOH has been associated with metastasis and there is evidence that 2 distinct breast tumor suppressor genes may reside at this locus. 11p15 has also been intensively investigated because of frequent LOH at this locus in a number of other human cancers including bladder, lung, ovarian, kidney, and testicular carcinomas (reviewed in 70). Several childhood tumors including Wilms' tumor, adrenocortical carcinoma, rhabdomyosarcoma, and hepatocellular carcinoma show specific loss of maternal 11p15 alleles, suggesting a role for genomic imprinting. Chromosome transfer experiments have also indicated a tumor suppressor gene resides at this locus, the WT2 gene involved in Wilms' tumor and possibly rhabdomyosarcoma (reviewed in 71), either of which could be due to loss of a Cdk inhibitor. In addition, rearrangements in the 11p15 region are found in Beckwith-Wiedemann Syndrome (BWS) which is characterized by numerous growth abnormalities, including macroglossia (enlarged tongue), gigantism, visceromegaly (enlarged organs) and an increased risk (7.5%) of childhood tumors (72). BWS occurs with an incidence of 1 in 13,700 births, 85% of which are sporadic and 15% familial (73). Genetic analysis indicates maternal carriers, also suggesting a role for genomic imprinting (reviewed in 74). Several features of KIP2 make it a reasonable candidate as a mediator of some phenotypes of BWS. First, a Cdk inhibitor could explain both overgrowth and tumorigenesis phenotypes. Furthermore, the expression pattern of KIP2 in mouse correlates with areas known to be affected in BWS including the tongue, kidney, muscle, and the eye. Third, KIP2 is imprinted and maternally expressed. Furthermore, LOH at 11p15 in Wilms' tumors are exclusively maternal, offering further support for the possibility that KIP2 might be the WT2 gene. LOH of the breast has not yet been examined for parental specificity of LOH. However, the potential for the existence of two tightly linked tumor suppressors (75) affecting the breast at 11p15 might complicate the analysis of parentally biased LOH depending on the relative frequency of the two events. Nevertheless, the biochemical properties of KIP2, its physical location and expression patterns suggest that it may be the tumor suppressor at 11p15.

The goals of our work were: 1) to determine whether p57 is imprinted in the breast, 2) to construct mice lacking p57, 3) to analyze the phenotype of mice lacking p57, 4) to analyze the role of the QT domain in p57 function by looking for binding proteins, and 5) to characterize the regulation of p57 and 6) to look for additional CKIs in the breast. To date, we have made significant progress on these initial goals. Our progress in these areas is summarized below.

## Body

### Aim 1: Determination of p57 imprinting status in the breast.

Imprinting patterns are conserved among tissues in mammals. We had originally intended to analyze p57 imprinting in human breast tissue. However, we were able to answer this question definitively in the mouse because of our success in making the knockout mouse for p57. As we have determined the p57 gene in the mouse is imprinted and expressed exclusively from the maternal chromosome just as it is in humans<sup>(76)</sup>. This was shown by looking at the protein found in mice lacking the maternal copy of p57. These heterozygous mutant embryos show no p57 mRNA staining at all in any part of the body including the breast (Fig. 1E). In addition, there is no p57 protein detected either (Figure 1C, E). This proves that p57 is imprinted in all tissues of the mouse including the breast. Therefore we have completed this Aim.

### Aim 2: Construction of mice deficient in KIP2.

#### Targeted Disruption of the Mouse p57<sup>KIP2</sup> Gene

A targeting construct that removed exons 1 and 2 (Fig. 1A) (87% of p57<sup>KIP2</sup>) was introduced into AB2.1 ES cells. G418/gancyclovir resistant cells were screened for homologous recombination by Southern-blot analysis (Fig. 1B). Homologous recombinant cells were injected into blastocysts from C57BL/6 mice and male chimeras were mated to C57BL/6 females. Germline transmission was confirmed by Southern blotting (Fig. 1B). F1 heterozygous animals were backcrossed to C57BL/6 mice to maintain the disrupted allele. To avoid ambiguity, we indicate the parental origin of alleles in heterozygotes by a superscript m for maternal or p for paternal.

The disrupted allele is a null as demonstrated by the absence of p57<sup>KIP2</sup> mRNA (Fig. 1E) and protein (Fig 1C, E) in p57<sup>-/-</sup> or p57<sup>+/-m</sup> animals. p57<sup>KIP2</sup> is detected in the tectum of brain, kidney, adrenal gland, muscle, lung, and cartilage in wild-type embryos. p21<sup>CIP1</sup> and p27<sup>KIP1</sup> levels were unchanged in tissues from p57<sup>KIP2</sup> mutants, except in muscle where a slight increase in p27<sup>KIP1</sup> was detected (Fig. 1C). p57<sup>+/-p</sup> animals had wild-type expression of p57<sup>KIP2</sup>.

#### Generation of p57<sup>KIP2</sup> Mutant Mice

Of 32 offspring from p57<sup>+/-p</sup> female to p57<sup>+/+</sup> male matings, no p57<sup>+/-</sup> animals were present when genotyped at two weeks of age (Table 1). Of 82 offspring from p57<sup>+/-p</sup> intercrosses, 55% were p57<sup>+/+</sup> and 45% were p57<sup>+/-</sup> (Table 1); no p57<sup>-/-</sup> animals survived to two weeks of age. Mendelian inheritance predicts a 1: 2 ratio for p57<sup>+/+</sup> to p57<sup>+/-</sup> animals, indicating half of the p57<sup>+/-</sup> animals died before genotyping. We observed dead or dying new-born mice in several litters, and these were found to be mutant. We concluded that p57<sup>KIP2</sup> is required for postnatal survival in a hybrid C57BL/6-129Sv background. Although lethal in this background, we recently discovered that crossing to the outbred CD1 strain allows some p57<sup>+/-m</sup> animals to survive well beyond day one.

When evaluated between E18.5 and E20, genotypes were detected at expected Mendelian frequencies (Table 1). However, 10% of mutant embryos were dead, staged from E13 to E16, consistent with the fact that maternal inheritance of the p57<sup>KIP2</sup> null allele is lethal (Fig. 1D). p57<sup>-/-</sup> animals are phenotypically indistinguishable from affected p57<sup>+/-m</sup> heterozygotes. Thus we have completely completed this Aim ahead of schedule.

### Aim 3: Analysis of p57 mutants animals.

We have completed an exhaustive analysis of the mutant phenotypes present in the p57 mutant animals. These are discussed below in the order in which we detected them.

#### Mice Lacking p57<sup>KIP2</sup> Have Omphalocele



Mutant embryos showed umbilical abnormalities as early as E16.5. A herniated abdomen was noticeable in all mutants (Fig. 2A, d&e) together with malrotation of the intestines resulting in placement of the jejunum and ileum in front of the liver. Occasionally, the small intestines were found outside the abdominal cavity (omphalocele), a range of phenotypes characteristic of BWS in humans (Fig. 2A, b). Most dead or dying neonates had a slit in their abdomen where the umbilicus is normally positioned, and portions of their visceral organs were missing, presumably devoured by their mother in the process of removing the placenta and yolk sac (Fig. 2A, c).

The small intestines develop outside the body till E15.5<sup>77</sup>, then enter the body cavity through a poorly defined mechanism. While it is unclear how omphalocele occurs in BWS, the prevailing assumption is that visceral overgrowth limits abdominal space. In p57<sup>KIP2</sup> mutant embryos, visceral overgrowth was not evident. p57<sup>KIP2</sup> is highly expressed in intestinal mesenteries which forms connections between the intestine folds and the abdomen and may participate in intestine re-entry. However, no obvious histological malformation was detected in mutant mesenteries.

### **Body Wall Muscle Dysplasia in p57<sup>KIP2</sup> Mutants Contributes to Omphalocele**

E18 mutants are 10% shorter than weight-matched p57<sup>+/+</sup> embryos (Fig. 3A, a). However, skeletons are nearly identical lengths (Fig. 3A, b), indicating a different etiology for the body length anomaly. Since proper musculature is required for skeletal stature, we investigated muscle organization. Histological examination of mutant embryos revealed defects in the position of body wall muscles (Fig. 3B, b&d). At the umbilicus level, the muscle did not reach as far towards the midline of the abdomen in mutants as in wild-type mice, leaving large areas of the abdominal wall uncovered by muscle. To distinguish whether this was a cause or consequence of herniation, we examined E14.5 embryos in which a physiological omphalocele is normally present. Muscle in the mutants also failed to reach the midline (Fig. 3B, a&c), strongly suggesting that the abdominal wall defect is responsible for the subsequent umbilical herniation.

Overall muscle differentiation was not affected in p57-mutant mice as evidenced by normal muscle fiber organization and peripheral location of nuclei. Surprisingly only 50% of muscle nuclei express p57<sup>KIP2</sup> (Fig. 3B, e and f). This is consistent with the two muscle lineages hypothesis, the MyoD and Myf5 lineages. Perhaps p57<sup>KIP2</sup>, which is also expressed in E11.5 somites (data not shown), is expressed in only one lineage and is required for muscle cell migration.

### **Cleft Palate May Contribute to Neonatal Lethality in p57<sup>KIP2</sup> Mutant Mice**

Cleft palate (secondary palate) and difficulty in breathing was noticeable in all mutant neonates (Fig. 2B, compare a&b). Milk was found in their lungs, and air in their stomach and intestines causing inflation and stretching. No histological abnormalities were found in mutant diaphragm, lung, bronchi, and trachea. The severity of cleft palate was variable but could compromise breathing by allowing an accumulation of liquid in the nasopharynx that is subsequently brought into the lungs by inhalation.

Secondary palate closure is a complex process<sup>78</sup> Vertically growing palate shelves elevate by E13 and grow together, fusing by E16 to form the secondary palate<sup>77</sup>. Analysis of E20 mutant embryos demonstrated that the palate failed to fuse, but maintained normal organization (Fig. 2B, c&d). p57<sup>KIP2</sup> is expressed in mesenchymal cells of the palate and muscle cells of the tongue, but not nasal or oral epithelia (Fig. 2B, e). Failure of palate closure could result from defects in mesenchymal cell migration, response to induction signals, cell proliferation, or increased apoptosis due to inappropriate proliferation. The tongue of mutant mice is not enlarged and is therefore not interfering in palate closure.

### **Renal Medullary Dysplasia in p57<sup>KIP2</sup> Mutants**

A major histopathological finding in BWS is non-cystic medullary dysplasia and enlargement of the kidney<sup>79</sup>. BWS medullary pyramids are frequently poorly formed and exhibit abundant connective tissue stroma with widely separated renal tubules<sup>80</sup>. In p57<sup>KIP2</sup> mutants, the size and organization of

the kidney was normal (Fig. 4 A-H), but the inner medullary pyramid was significantly smaller than normal. Normally at E18.5 and E20, the maturing glomeruli are juxtamedullary and nephrons have developed long loops of Henle reaching deep into the inner medullary region (Fig. 4G). However, mutants have fewer renal tubules (loops of Henle and collecting ducts) and more stromal cells in the inner medulla (Fig. 4D&H), a phenotype very similar to that of BWS patients<sup>79</sup>. No defects in primary nephrogenesis were observed, indicating that ureter bud branching and mesenchymal induction occurred normally in the mutant kidney.

To determine if medullary dysplasia is due to improper development or to regression of a properly formed kidney, a developmental analysis was performed. At E16 the renal pelvis forms through fusion of initial ureter branches. A normal renal pelvis was observed in the E16.5 mutant kidney (Fig. 4A&B). At this stage the inner medulla normally begins to form, but in the mutant this was totally absent. At E18.5, the mutant inner medulla has also failed to develop properly (Fig. 4C&D). This indicates the medullary dysplasia is due to arrested or improper development.

p57<sup>KIP2</sup> is expressed in podocytes of maturing glomeruli and in interstitial stromal cells between renal tubules (Fig. 4I) but not the tubules themselves. This expression pattern argues against an intrinsic defect in the renal tubule, and underscores the importance of tissue-tissue interactions between epithelia of renal tubules and surrounding mesenchymal cells during the development of Henle's loops, as has been previously demonstrated for ureteric branching and the mesenchymal to epithelial transition.

### **p57<sup>KIP2</sup> is Required for Endochondral Bone Development and Expression of Collagen X**

Skeletal staining with alcian blue (cartilage) and alizarin red (bone) revealed abnormalities in mutant skeletons. At E14.5, the two sternal bands in mutants were widely separated, while those in wild-type animals had already fused (Fig. 5A, a&b). By E19, wild-type sternbrae were fully ossified, whereas ossification had just begun in the mutant (Fig. 5A, c&d). Two separate ossification centers in each sternbra were clearly seen in the mutant, indicating imperfect fusion of sternal bands (Fig. 5A, d). Smaller ossification centers were observed in mutants at all stages of development in forelimbs, vertebra, and supraoccipital bone as shown in Fig. 5A, e-l. While mutant limbs are shorter, they are thicker than the wild-type (compare Fig. 5 g and h).

Vertebrate long bones are formed through endochondral ossification which involves formation of a cartilage framework that is converted to bone by replacement. Within this framework, cells are organized into distinct zones; the epiphyseal center zone contains resting chondrocytes (rc) that act as stem cells for the adjacent proliferative zone (pc) where chondrocytes proliferate and form columns, and the hypertrophic zone (hc) containing dying chondrocytes that are in the process of ossification. Histological examination of mutant long bone sections showed a slight disorganization of the columnar alignment of differentiating chondrocytes (Fig. 5B, a&b). Furthermore, the mutant hypertrophic zone is slightly thinner than wild-type, containing smaller cells, suggesting impairment of chondrocyte differentiation.

p57<sup>KIP2</sup> is expressed at moderate levels in resting chondrocytes, low levels in the proliferative zone and very high levels in the hypertrophic zone (Fig. 5B, c). We examined cell cycle withdrawal in the mutant hypertrophic zone to determine if cell division in this zone may account for resistance to ossification. A higher level of BrdU incorporation was observed in the resting (2.2-fold) and proliferative (1.6-fold) chondrocytes of E15 mutant animals, but at later times such as P0 only a small increase in BrdU labeling was observed in resting (20%) and proliferative (14%) chondrocytes. At day 18.5 there is a 10% greater cell density in these two zones, which may explain the bone thickening in mutant animals (Fig. 5 e-h). However, no BrdU labeling was observed in either the mutant or wild-type hypertrophic zones at any stage.

Collagen X is expressed in hypertrophic chondrocytes and has been implicated in proper bone development<sup>81</sup>. Collagen X expression was significantly reduced in the mutant hypertrophic zone (Fig. 5B, d&e). Thus, p57<sup>KIP2</sup> is required for expression of collagen X and possibly other genes that facilitate the ossification of chondrocytes. Since no cell cycle entry was observed in mutant

hypertrophic chondrocytes, the failure to express collagen suggests that p57<sup>KIP2</sup> may play a direct role in processes of differentiation.

#### **Adrenal Cortex Hyperplasia and Cytomegaly in p57<sup>KIP2</sup> Mutants**

The adrenal gland is among the most consistently enlarged organs in BWS patients and shows extensive cytomegaly. p57<sup>+/-m</sup> mutants show a significant enlargement of the adrenal gland (25% to 100% increase in volume, Fig. 6A). While mutant adrenals were normal histologically, there was a 3-fold increase in the frequency of cytomegaly (Fig. 6C, D). p57<sup>KIP2</sup> expression is restricted to fetal adrenal cortex (Fig. 6B), indicating a role in controlling cell proliferation.

#### **Loss of p57<sup>KIP2</sup> Results in Increased Cycling and Apoptosis in the Mouse Lens.**

p57<sup>KIP2</sup> protein is present in high levels in postmitotic lens fiber cells, with low level, sporadic expression in the anterior epithelial layer (Fig. 7 A, B). p57<sup>KIP2</sup> is induced in the equatorial zone (Fig. 7A, arrows) where epithelial cells are withdrawing from the cell cycle and initiating terminal differentiation<sup>82</sup>, suggesting a role for p57<sup>KIP2</sup> in promoting one or both of these processes. Analysis of p57<sup>-/-</sup> and p57<sup>+/-m</sup> lenses revealed grossly normal lens structure at E13.5 with minor vacuolization, but a more pronounced vacuolization in E15.5 and older lenses (Fig. 7E). Late-stage differentiation markers such as  $\gamma$ -crystallin and membrane-intrinsic protein-26 were unaffected (data not shown). However, p57<sup>KIP2</sup> loss causes inappropriate S phase entry in lens fiber cells (Fig. 7, +/+ F, -/- G) and an increase in apoptotic nuclei (Fig. 7I and J) possibly contributing to the vacuolated appearance. Another phenotype of p57<sup>-/-</sup> lenses was a 10-fold increase in apoptosis in the anterior epithelial compartment (Fig. 7J). Normally, epithelial cells that accumulate in the central-most position of the anterior epithelial layer are eliminated by a p53-independent apoptotic mechanism to maintain the single cell layer anteriorly<sup>83</sup>. Since these cells express p57<sup>KIP2</sup>, the increase in apoptosis could result from an accelerated rate of proliferation and execution of a normal mechanism designed to eliminate excess cells in the central zone of the anterior lens.

Thus we have provided an extensive analysis of the progress in the analysis of the phenotypes associated with the loss of p57 in the mouse and have completed this part of the aim. An additional portion of this aim is to look at the phenotypes associated with the loss of multiple inhibitors. Toward this end we have begun to mate the p57 deficient mice to p21 and p27 deficient animals in order to make double mutant mice.

#### **Aim 4. Analysis of the QT domain.**

CKIs of the p21 family have multiple domains. For example, in addition to Cdks, p21 can bind PCNA and this interaction inhibits PCNA-dependent DNA replication<sup>(67, 68)</sup> making p21 a dual specificity inhibitor. Importantly, p21 can associate with PCNA and Cdks simultaneously and may serve to target active kinases to particular substrates. Human p57 also has multiple domains: An N-terminal Cdk-binding domain, a proline alanine-rich central domain called the PAPA repeat, and a C-terminal sequence, the QT domain, displaying 50% identity with the C-terminus of p27. In the mouse p57 gene, the central region has both a proline rich region and an acidic repeat region. With the exception of the inhibitory domain, the function of these additional domains are not known. However, the finding of strong conservation in the QT-domain with p27 suggests that it has roles independent of Cdk binding or inhibition. One possible function for this domain is to recruit proteins to the cyclin/Cdk complex. We are planning to look for proteins that bind to C-terminus of p57 by the two hybrid system. We are also interested in over expressing that domain of p57 in transgenic animals. We have not initiated these studies yet.

#### **Aim 5. Transcriptional control of p57<sup>KIP2</sup>.**

Through in situ analysis and immunohistochemistry we know where p57 is expressed (Figure 1). We propose to perform an analysis of the regulatory sites in the p57 promoter that controls its expression. We also proposed to sequence the promoter of p57 to provide an initial characterization

of the locus. We have made some initial progress on this aim. We have isolated the promoter DNA and have begun sequencing it. However, we have only just begun this and hope to have it completed and analyzed during the next year. Thus, this aim is only now being started.

**Aim 6. Identification of new CKIs and other potential regulators of Cdks from normal breast.** This aim proposes to look for additional Cdk binding proteins in the breast using the two hybrid system and breast cDNA libraries. We have the cDNA libraries but have not begun the screens yet because we have been so busy analyzing the mice. Hopefully we can begin these interesting experiments during the next funding period.

## **Conclusion:**

The last year was a very productive one for our lab and the cell cycle field in general. Our work funded under this grant allowed us to establish the role of p57<sup>KIP2</sup> in mouse development and the human cancer and overgrowth syndrome BWS. p57<sup>KIP2</sup> clearly acts as a regulator of cell proliferation in the adrenal gland, the lens epithelia, and certain chondrocytes. The partial dependency on p57<sup>KIP2</sup> for reducing cell proliferation reveals the redundant mechanisms used to limit tissue growth. A similar situation is observed in cell culture where agents that induce cell cycle arrest immediately increase levels of certain CKIs and subsequently reduce the levels of the cyclins and Cdks. While undergoing the process of reducing Cdk activity during differentiation, the absence of CKIs may allow additional cell cycles to occur before Cdk activity is sufficiently reduced to block cell cycle entry.

Rb loss is associated with increases in proliferation, impaired expression of differentiation markers, and inappropriate apoptosis in lens fiber cells<sup>83</sup>. p57<sup>-/-</sup> lenses show less cell proliferation and apoptosis than Rb<sup>-/-</sup> lenses, thus p57<sup>KIP2</sup> is likely to play a partially redundant role upstream of Rb. However, the increase in apoptosis in the anterior epithelial compartment is greater in p57<sup>-/-</sup> than Rb<sup>-/-</sup> lenses. Thus, even in the same cell type, the relationship between p57<sup>KIP2</sup> and Rb (and possibly other cell cycle regulators) changes with respect to differentiation state. Furthermore, the ossification defect in p57<sup>-/-</sup> mice is similar to that observed in p107<sup>-/-</sup>-p130<sup>-/-</sup> double mutants<sup>84</sup>, and it is likely that p57<sup>KIP2</sup> is also regulating these proteins to control proliferation in chondrocytes.

Several tissues in p57<sup>-/-</sup> mutants showed developmental defects not obviously directly linked to increased cell proliferation, e.g. defects in kidney development, differentiation of hypertrophic chondrocytes, muscle organization and formation of the secondary palate. These defects may indicate roles in differentiation distinct from p57<sup>KIP2</sup>'s biochemical role as a CKI. In the case of the ossification defects, the hypertrophic chondrocytes exit the cell cycle properly but were disorganized and failed to express differentiation markers such as collagen X. Non-Cdk binding regions of the p57<sup>KIP2</sup> protein, such as the repeat regions or the conserved QT domains, may play cell cycle-independent roles in differentiation.

The p57<sup>KIP2</sup>-defective mouse provides an unambiguous assessment of the role of p57<sup>KIP2</sup> in BWS. Its BWS-related phenotypes support a causal role for the mutation found by Hatada et al.<sup>85</sup>. Both patients harboring p57<sup>KIP2</sup> mutations displayed omphalocele, macroglossia, gigantism and earlobe grooves. p57<sup>KIP2</sup>-deficient mice also show omphalocele but not gigantism, macroglossia, visceromegaly, and hypoglycemia (data not shown). However, the expression pattern of p57<sup>KIP2</sup> is consistent with a possible role in both gigantism and macroglossia; the mouse tongue expresses extremely high levels of p57<sup>KIP2</sup> during development, as does the human placenta which controls nutrient flow to embryos.

It is unknown which of the internal manifestations of BWS these two patients exhibit. However, the p57<sup>-/-</sup> mouse displays several common internal BWS phenotypes including enlargement of the adrenal cortex, adrenal cytomegaly, and renal medullary dysplasia that are now strongly predicted to be present in the two affected individuals. Phenotypes present in the p57<sup>-/-</sup>



mouse not frequently considered to be associated with BWS are cleft palate, endochondrial ossification defects, and ocular lens impairment. It should be noted that unlike the mouse, human p57<sup>KIP2</sup> imprinting is incomplete with 5% residual expression from the paternal allele<sup>15</sup>. Therefore while mice lacking the maternal allele are nulls, the equivalent humans are hypomorphs. This may affect the relative penetrance of phenotypes between species. Nevertheless, increased frequency of cleft palate<sup>86,87</sup>, skeletal anomalies<sup>88</sup>, and cataract formation<sup>88</sup> have been reported in conjunction with BWS. Interestingly, Takato et al.<sup>87</sup> observed a very high frequency of palate defects including cleft and soft palate in one BWS study (6 of 10 patients) from Japan. The high frequency of palate defects in that study relative to others may reflect the differences in frequencies of modifier genes in distinct populations. In response to this study it was learned that BWS patient 6 from Hatada et al.<sup>85</sup> also had cleft palate (I. Hatada, personal communication). This provides further compelling support for p57<sup>KIP2</sup> involvement in BWS. Beckwith also observed that the long bones in BWS patients often show widened metaphyses and a thickened bony cortex<sup>89</sup>. This is consistent with our observations of the thickened bones in p57-deficient mice. Thus, the analysis of the p57<sup>KIP2</sup>-deficient mouse has provided not only proof of the role of p57<sup>KIP2</sup> in BWS, but has also extended the range of phenotypes expected upon further analysis of BWS patients with p57<sup>KIP2</sup> mutations. Furthermore, it has provided an explanation for the physiological basis of several phenotypes observed in BWS patients including skeletal abnormalities (chondrocyte proliferation and differentiation), omphalocele (abdominal muscle developmental defects) and adrenomegaly (absence of a cell proliferation inhibitor).

While the fact that the mice lacking p57 die soon after birth in our genetic background has hindered our ability to determine whether p57 is involved in breast cancer using a mouse model, we now know that p57 is a gene possibly involved in human cancer because of its association with BWS. We now know that humans lacking p57 exist and this knowledge should allow us to determine whether it is truly a tumor suppressor gene and whether it is involved in breast cancer in the coming years.

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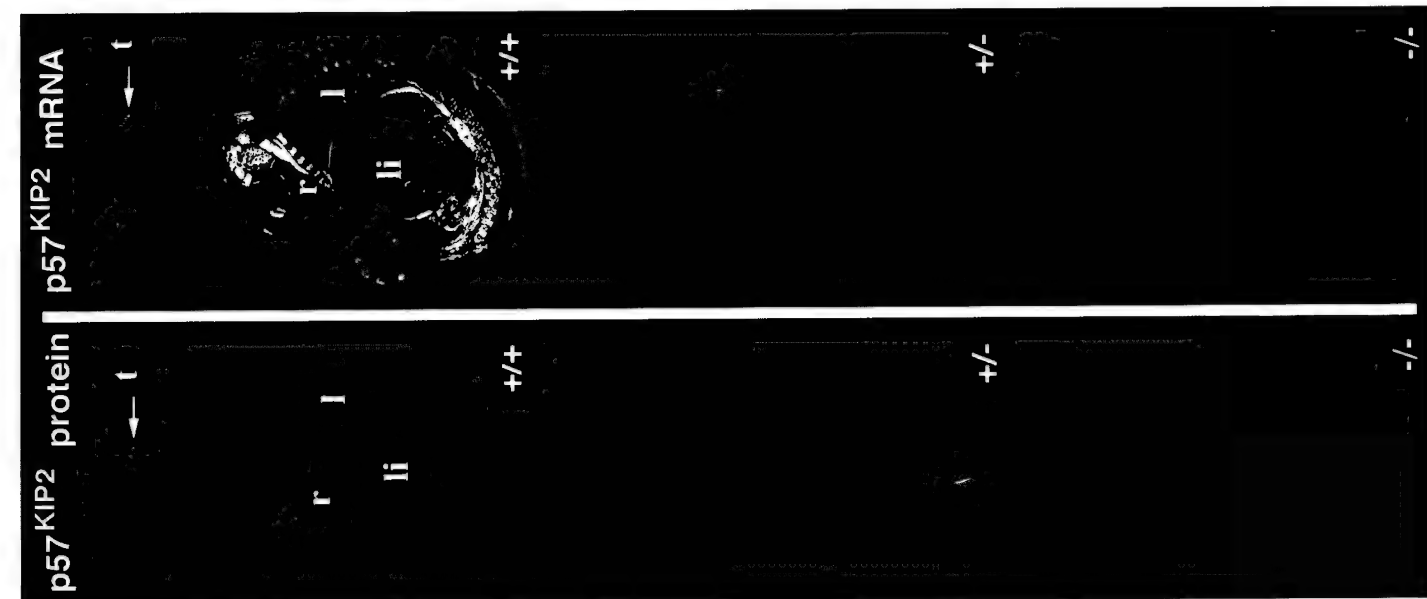


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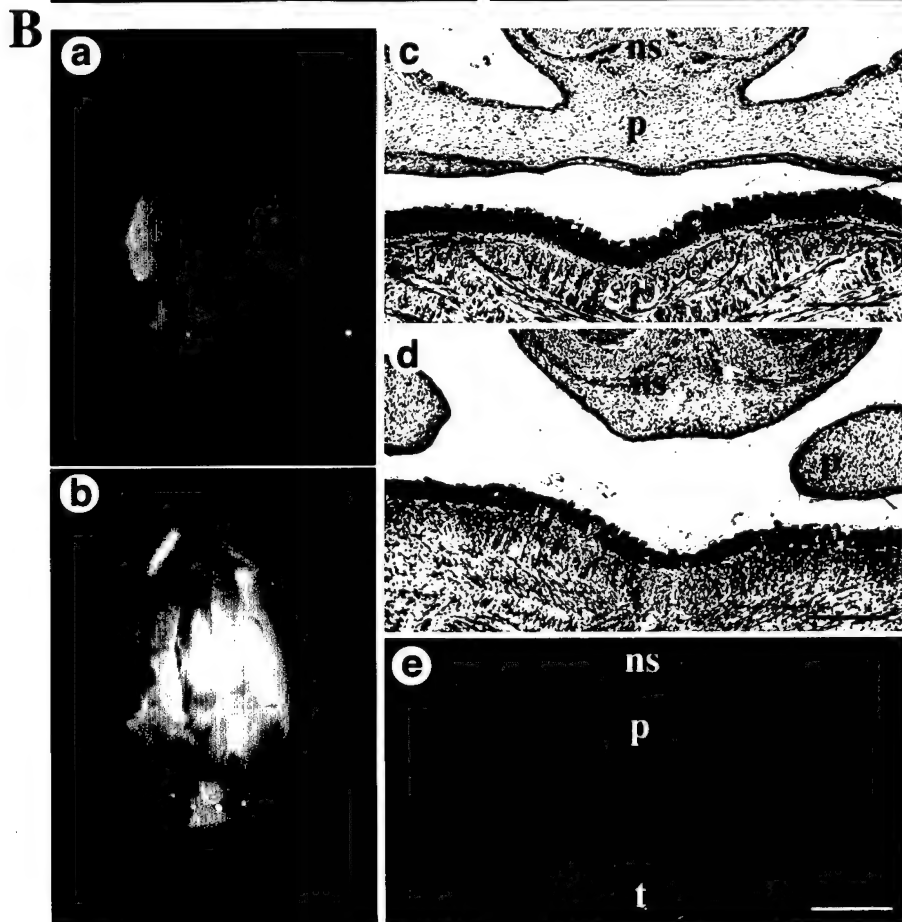
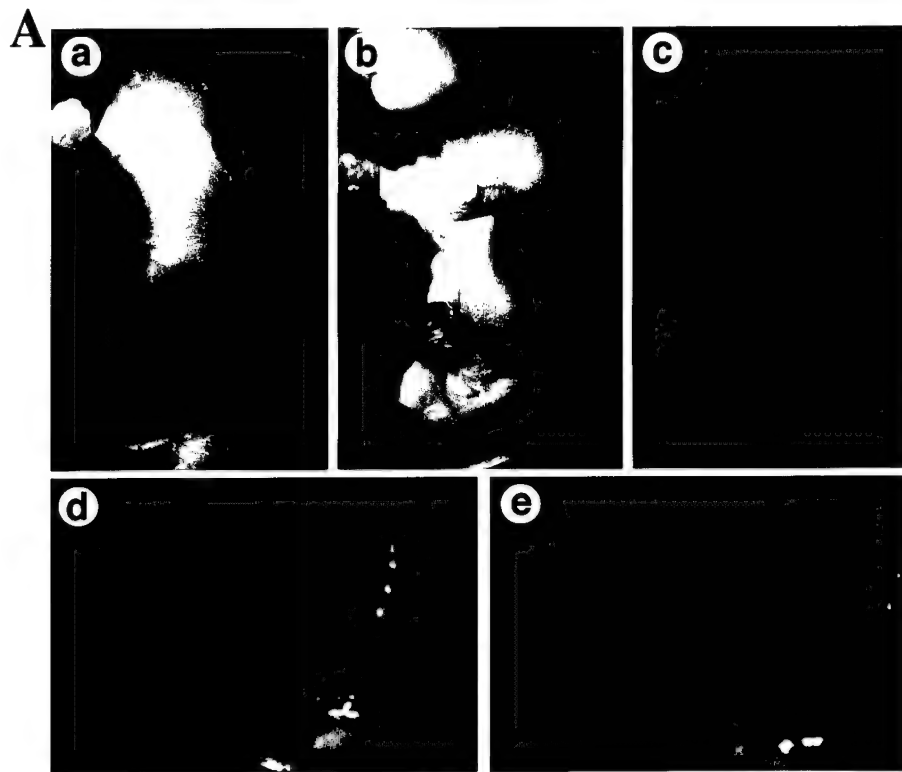


## Appendix

### Figure Legends

**Figure 1** Targeted disruption of p57<sup>KIP2</sup>. (A) p57<sup>KIP2</sup> disruption strategy. Probes (A and B) for Southern analysis are indicated. (B) Southern blot analysis of DNA from wildtype and mutant ES clones and embryos. (C) Western blot analysis of p57<sup>KIP2</sup>, p27<sup>KIP1</sup> and p21<sup>CIP1</sup> proteins in muscle and kidney. \* represents a form of p57<sup>KIP2</sup> resulting from phosphorylation or alternative splicing. (D) A pedigree analysis; squares are males; circles, females. Heterozygotes are represented as half-filled symbols. Animals displaying a mutant phenotype are indicated by slashes. (E) In situ hybridization and immunofluorescent analysis of sagittal sections derived from E15.5 p57<sup>+/+</sup>, p57<sup>+/-m</sup>, and p57<sup>-/-</sup> embryos. A combined image of p57<sup>KIP2</sup> protein (green) and nuclei stained with Hoechst dye (blue) is shown. l, lung; li, liver; r, rib; t, tectum.

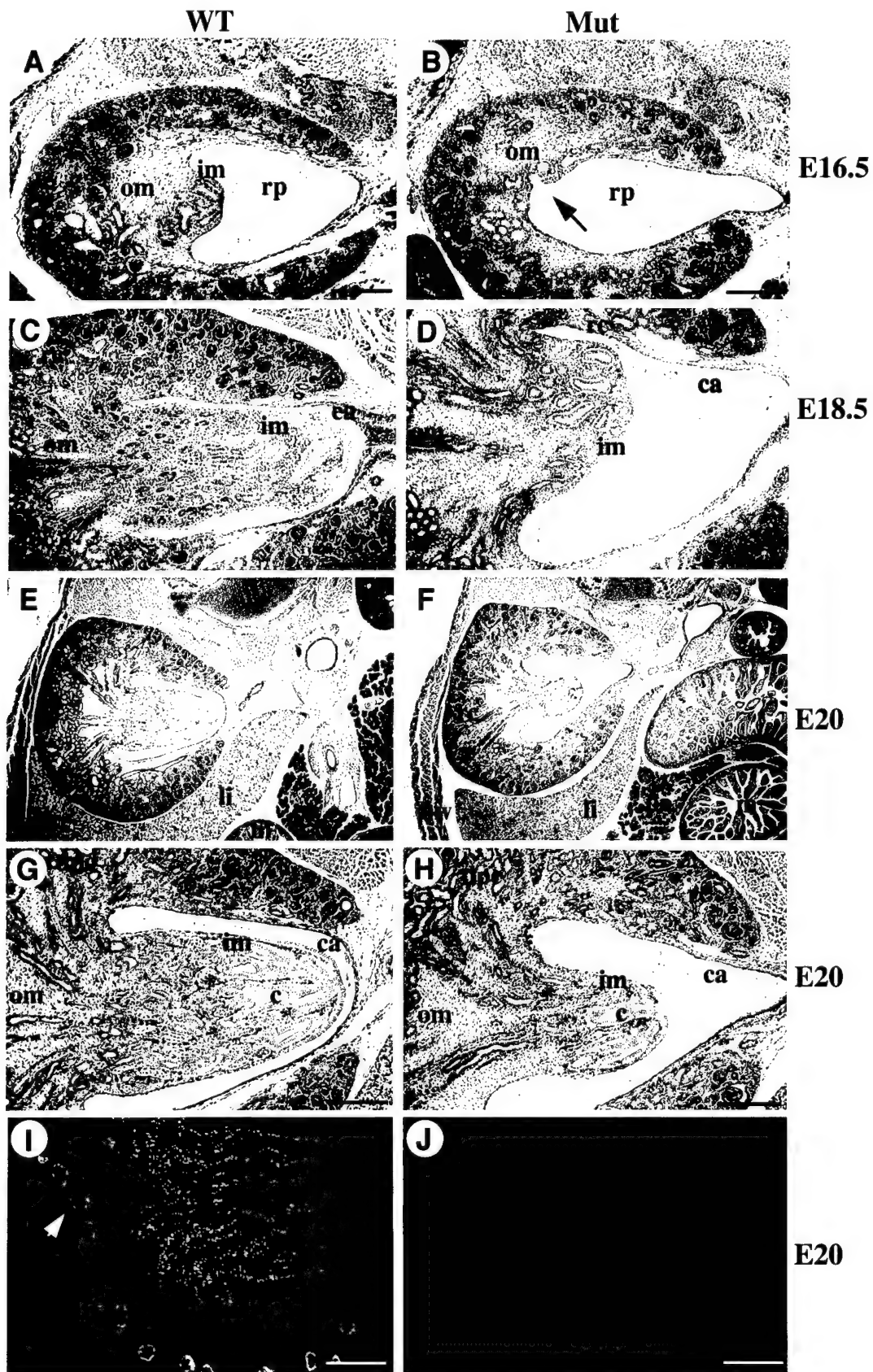




**Figure 2** Omphalocele, umbilical hernia and cleft palate in p57<sup>KIP2</sup> mutant mice. (A) Intestines are found outside the abdominal cavity in some p57<sup>KIP2</sup> mutant newborns (b) but not wildtype mice (a). Umbilical sacs were removed in (b) to expose intestines. Typically, dead neonates (mutants) had a slit on their abdomen with missing viscera (c). Umbilical hernia is observed in mutants (e, arrow) but not in wildtype littermates (d). a and b, E20; c, P0; d and e, E18. (B) Jaws were removed from P0 wildtype (a) and mutant (b) mice to allow palate viewing. Hemotoxylin and eosin stained coronal sections show a fused palate in E20 wildtype (c) mice and cleft palate in mutant (d). p57<sup>KIP2</sup> expression (green) in the palate mesenchyme and tongue muscle of an E20 embryo (e). ns, nasal septum; p, palate; t, tongue. scale bar, 200  $\mu$ m.

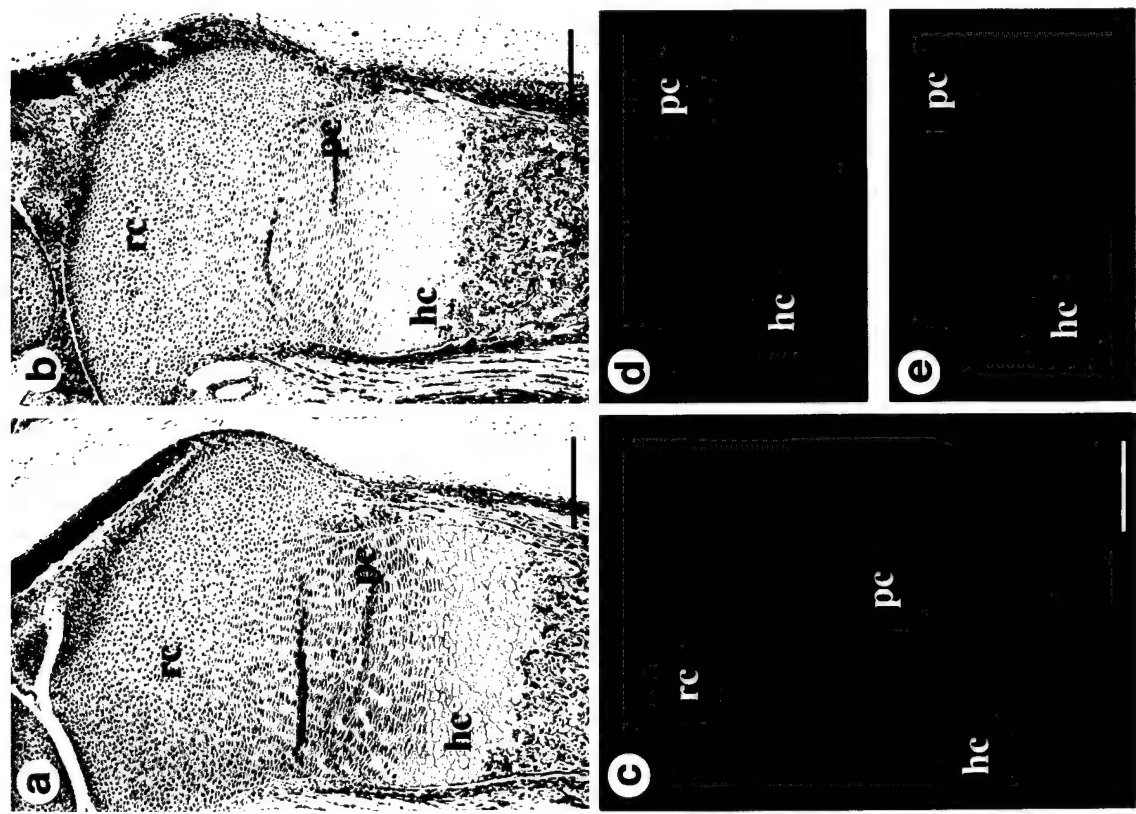
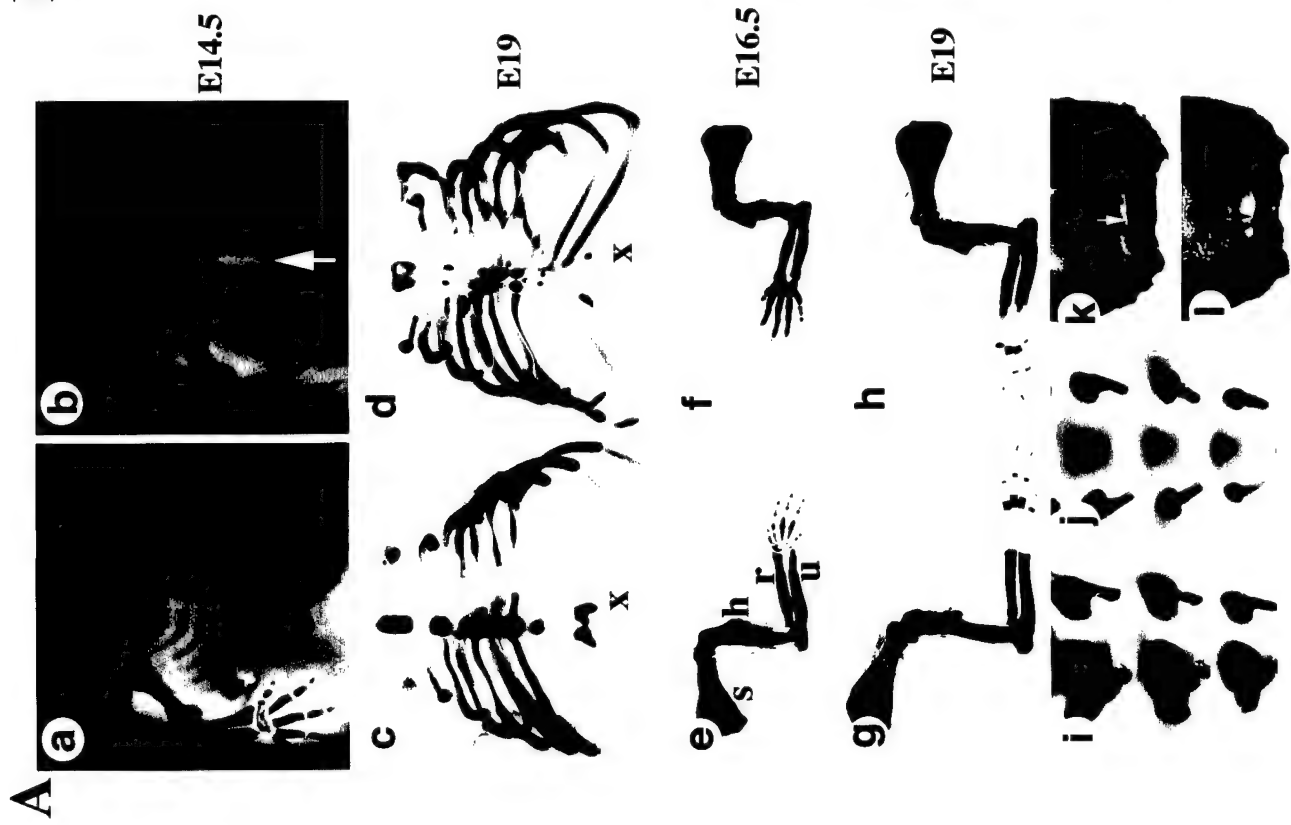


**Figure 3** Body wall dysplasia in p57<sup>KIP2</sup> mutants. (A) E18 p57<sup>+/+</sup>(wt) and p57<sup>+/-m</sup>(mut) embryos (a) and skeletons (b) prepared from the same embryos. Mutants have shorter bodies but normal skeletal length. (B) Transverse embryo sections were stained with hemotoxylin and eosin. The distance between umbilicus and the tip of rectus abdominis muscle (arrows) is greater in mutants than in wildtype littermates in E14.5 (a and c) and E18 (b and d) embryos. Skin development is also delayed in mutants (asterisks). p57<sup>KIP2</sup> protein was detected in the rectus abdominis muscle, connective tissue, skin, and liver (e), and is present in half of the nuclei in muscle (f); in, intestine; li, liver; m, muscle; um, umbilical cord. Scale bar, 200 $\mu$ m.

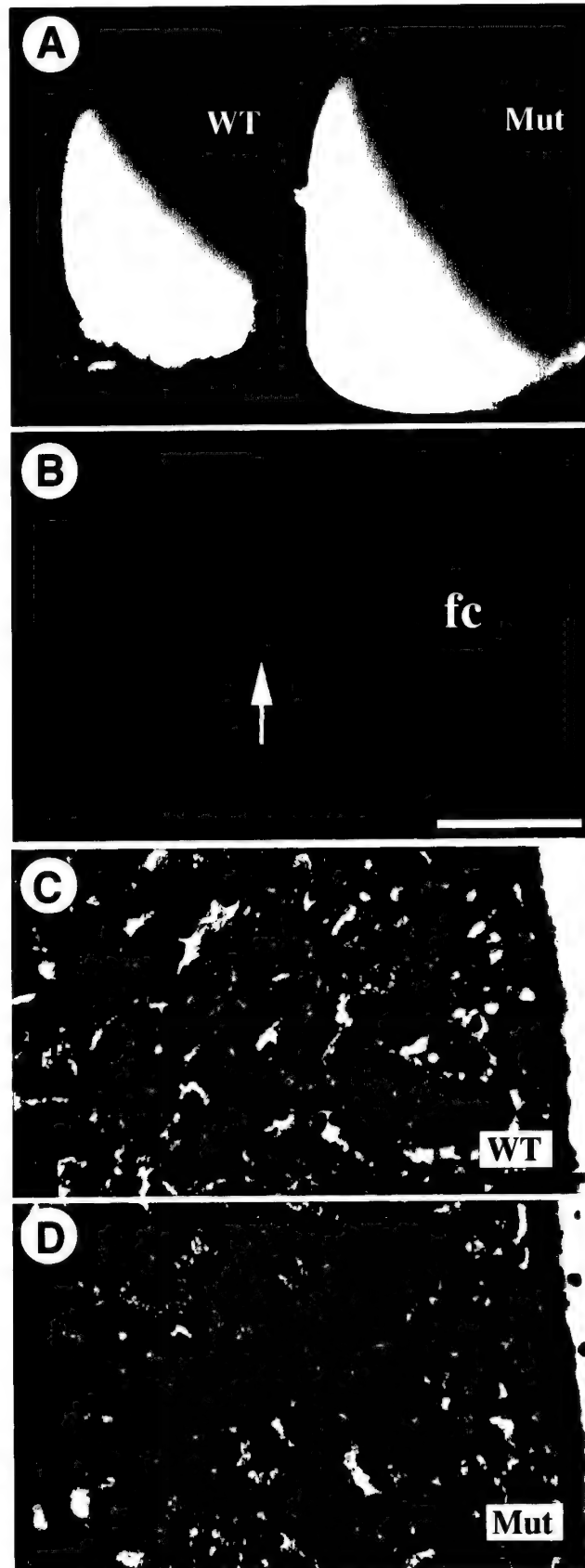


**Figure 4** Kidney medullary dysplasia in  $p57^{KIP2}$  mutants. (A and B) At E16.5, the inner medulla is absent in the  $p57^{-/-}$  mutant kidney (arrow). (C and D) At E18.5, a delayed elongation of the inner medulla is evident in the mutant kidney. (E and F) Low magnification shows relatively normal organization of the mutant kidney at E20. (G and H) A higher magnification of E and F. The mutant inner medulla is significantly smaller than the wildtype, containing fewer loops of Henle (\*) and collecting ducts with more stromal cells in between. (I and J).  $p57^{KIP2}$  protein is expressed in mesenchymal cells between renal tubules of the inner medulla and in podocytes of glomeruli (arrow), but not in mutants (J). bw, body wall; c, collecting ducts; dpc, distal and proximal convoluted tubules; im, inner medulla; in, intestine; li, liver; om, outer medulla; pa, pancreas; ca, renal calyx; rc, renal cortex; rp, renal pelvis. Scale bar, 200  $\mu$ m.

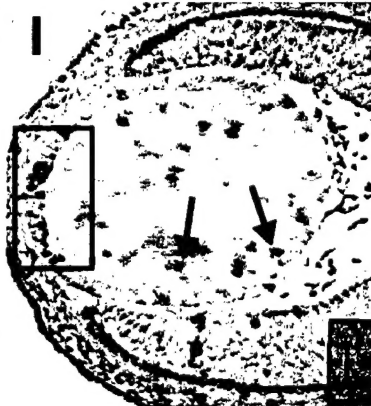
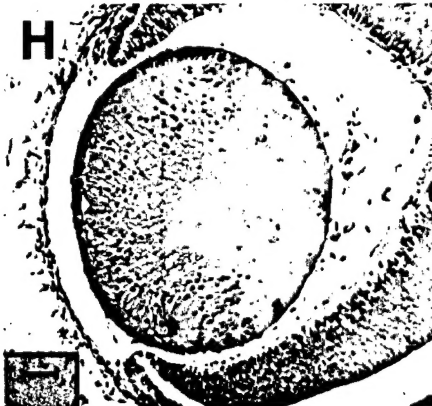
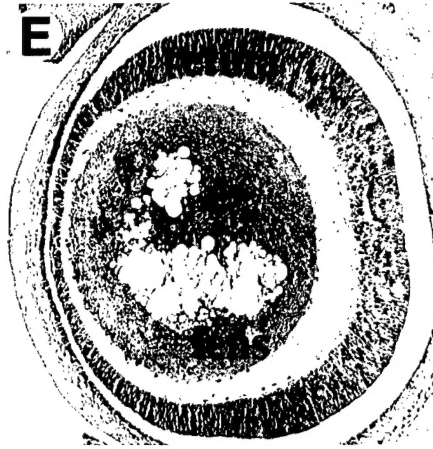
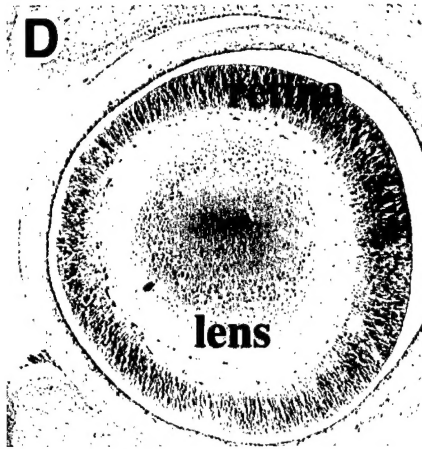
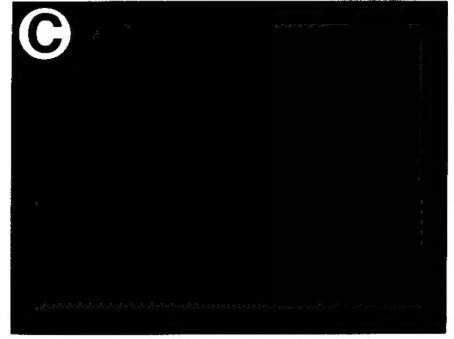
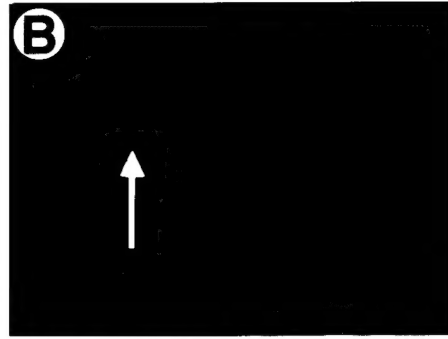
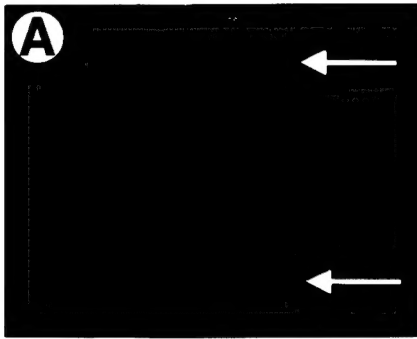




**Figure 5** Impaired endochondral ossification in  $p57^{KIP2}$  mutants. (A) Alcian blue (cartilage) and alizarin red (bone) staining of E14.5 skeletons showing fused sternum rudiments in wildtype (a) and an unfused sternum in  $p57^{+/-m}$  mice (b, arrow). Sternebrae are fully ossified in E19 wildtype (c) but not mutant (d) embryos. Note the separate sternum ossification centers and enlargement of the xiphoid process in (d). (e and f) Forelimbs of E16.5 (e, f) and E19 (g, h) wildtype (e, g) and mutant (f, h) embryos. Reduced ossification is observed in mutant E19 vertebrae (j) and P0 supraoccipital bone (l) relative to wildtype (i, k). (B) Longitudinal sections through the tibia of E18.5 wildtype (a) and mutant (b) embryos, stained with hematoxylin and eosin.  $p57^{KIP2}$  expression was visualized by immunofluorescence (c). Collagen X expression was detected in wildtype (d) but not in mutant (e) hypertrophic chondrocytes. h, humerus; hc, hypertrophic chondrocytes; pc, proliferating chondrocytes; r, radius; rc, reserving chondrocytes; s, scapula; su, supraoccipital bone; u, ulna; x, xiphoid process. Scale bar, 200  $\mu$ m.



**Figure 6** Hyperplasia of the adrenal gland in  $p57^{KIP2}$  mutants. (A) The E19 adrenal gland is enlarged in  $p57^{+/-m}$  mice. (B)  $p57^{KIP2}$  protein is expressed in the fetal cortex but not medulla (arrow) of an E20 adrenal gland. (C and D) Hematoxylin and eosin stained sections of E20 wildtype (C) and  $p57^{+/-m}$  (D) adrenal glands. Arrows indicate cytomegaly. fc, fetal cortex; m, medulla. Scale bar, 200  $\mu$ m in (B), 50  $\mu$ m in (C) and (D).



**Figure 7** p57<sup>KIP2</sup> loss causes increased proliferation and apoptosis in the lens. Panels A-C. Immunofluorescent analysis shows strong p57<sup>KIP2</sup> expression in all nuclei of the postmitotic lens fiber cell compartment (arrows) (A), occasional nuclear and cytoplasmic staining in anterior epithelial cells (arrow) (B), and no expression in p57<sup>-/-</sup> lenses (C). Panels D-E. Histological presentation of hematoxylin and eosin stained E15.5 lens sections (sagittal) derived from p57<sup>+/+</sup> (D) or p57<sup>-/-</sup> (E) embryos. Panels F-G. BrdU incorporation assays on E13.5 lens sections derived from p57<sup>+/+</sup> (F) or p57<sup>-/-</sup> (G) embryos. Brackets show inappropriate S phase entry in normally postmitotic lens fiber cells of the equatorial region. The arrow indicates S-phase entry in central lens fiber cells. Panels H-J. TUNEL assays on E13.5 lens sections derived from p57<sup>+/+</sup> (H), p57<sup>-/-</sup> (I), p57<sup>-/-</sup> embryos (J, higher magnification of the boxed region in panel I showing abundant apoptotic nuclei in the anterior epithelial layer). Scale bar, 10μm in F, G, H, I and 4μm in J.



**Table 1 Genotype frequencies of offspring.**

**A. Male p57<sup>+/+</sup> x Female p57<sup>+/-P</sup>**

p57 genotype	+/+	+/-
Number	32	0
% Observed	100	0
% Expected	50	50

**B. Male p57<sup>+/-P</sup> x Female p57<sup>+/-P</sup>**

p57 genotype	+/+	+/-	-/-
Number	45	37	0
% Observed	55	45	0
% Expected	25	50	25

**C. Male p57<sup>+/+</sup> x Female p57<sup>+/-P</sup>**

p57 genotype	+/+	+/-
Number	24	27
% Observed	47	53
% Expected	50	50

**D. Male p57<sup>+/-P</sup> x Female p57<sup>+/-P</sup>**

p57 genotype	+/+	+/-	-/-
Number	16	27	14
% Observed	28	47	25
% Expected	25	50	25

A and B were genotyped at 2 weeks of age.  
C and D were genotyped between E18.5 and E20.

## Personnel

Stephen J. Elledge  
J. Wade Harper  
Pumin Zhang  
Calvin Wong

## Publications

- Zhang, P., Liegeois, N.J., Wong, C., Hou, H., Finegold, M., DeMayo, F., Silverman, A., Harper, J.W., DePinho, R.A., and Elledge, S. J.(1997). Altered cell differentiation and proliferation in mice lacking p57<sup>KIP2</sup> indicates a role in Beckwith-Wiedeman Syndrome. *Nature* 387:151-158.
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